cis-Inhibition of proteasomal degradation by viral repeats: impact of length and amino acid composition

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Abstract The Gly–Ala repeat (GAr) of the Epstein–Barr virus nuclear antigen 1 is a *cis* acting inhibitor of ubiquitin–proteasome proteolysis. We have investigated the capacity of various repeats to inhibit the turnover of the proteasomal substrate IκBα. Inhibition of TNFα-induced degradation was achieved by insertion of octamers containing three alanines or valines, interspersed by no more then three consecutive glycines. The inhibitory activity was abolished by increasing the length of the spacer, by eliminating the spacers, or by substitution of a single hydrophobic residue with a polar or charged residue. A serine containing octamer was inactive but inhibition was partially restored by insertion of three consecutive repeats. These findings suggest a model where inhibition requires the interaction of at least three alanine residues of the GAr in a β-strand conformation with adjacent hydrophobic binding pockets of a putative receptor.

Key words: Ubiquitin–proteasome pathway; Epstein–Barr virus; EBNA-1; Gly–Ala repeat

1. Introduction

Ubiquitin–proteasome dependent proteolysis is involved in the regulation of cellular processes such as cell cycle progression, transcription, tissue development and atrophy, flux of substrates through metabolic pathways, selective elimination of abnormal proteins, and processing of intracellular antigens for presentation to MHC class I restricted CTLs (reviewed in [1,2]).

Intracellular parasites, such as viruses, have developed strategies to interfere with this proteolytic machinery by interacting with various components of the pathway [3–12]. A particularly interesting example of a viral protein that targets the ubiquitin–proteasome pathway is the Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA1). EBNA1 contains an internal repeat exclusively composed of glycines and alanines (GAr) that inhibits in *cis* the presentation of endogenous antigens to MHC class I restricted cytotoxic T-lymphocytes (CTLs) [13] and prevents ubiquitin–proteasome dependent proteolysis in vitro [14]. EBNA1 is the only EBV protein that is ubiquitously expressed in all EBV-associated malignancies [15], and its resistance to CTL recognition could play an important role in the maintenance of EBV latency. Interestingly, proteins from other human and primate γ-herpesviruses, such as the EBNA1 homologs of the baboon and rhesus monkey lymphocryptoviruses (BaLCV and RhLCV) [16], and the latency-associated nuclear antigen (LANA) of Kaposi sarcoma herpes virus (KSHV) [17,18], also contain repetitive domains of different lengths and sequences, suggesting that the repeats may play a common role in the biology of these viruses.

The mechanism whereby the GAr influences ubiquitin–proteasome dependent proteolysis is not fully understood. Using as indicator IκBα, a known target of this proteolytic pathway [19], we have identified an eight amino acid long GAr polypeptide, GGAGAGAG, as the minimal sequence required for the inhibitory effect [20]. An IκBα chimera containing the repeat was phosphorylated and ubiquitinated in response to tumor necrosis factor α (TNFα), but was then released from complexes with NF-κB and failed to associate with the proteasome. In addition, insertion of this inhibitory sequence in different positions of IκBα decreased its basal turnover in vivo, resulting in constitutive dominant-negative mutants.

In the present study, we have investigated the relationship between the inhibitory activity and the amino acid composition of the repeat. We demonstrated that inhibitory repeats contain at least three hydrophobic amino acids interspersed by no more then three consecutive glycine residues. The inhibitory activity was abolished by substitution of a single hydrophobic residue of the octamer with the polar amino acid serine but the effect could be partially compensated by increasing the length of the repeat.

2. Materials and methods

2.1. Plasmids

The strategy for construction of IκBα chimeras carrying N-terminal repeats was previously described [20]. Briefly, a human IκBα gene tagged with three influenza virus hemagglutinin epitopes (HA) [21,22] was cloned in the *Hin*dIII and *Xba*I sites of the pSP64-polyA vector (Promega). The *Not*I–*Xba*I region of the vector downstream of the IκBα stop codon was substituted with the *Not*I–TAG–*Xba*I linker sequence to remove the *Sal*I site located in the polylinker. Synthetic oligonucleotides with 3′ overlapping ends encoding GAr sequences and substitution analogues were annealed at 42°C in 10 mM $MgCl_2$, 20 mM Tris–HCl pH 8.0, 2 mM DTT. The complementary strands were synthesized by Klenow enzyme. The resulting DNA fragments were digested with *Sal*I and *Xho*I and cloned into the remaining unique *Sal*I site located between the start codon of IκBα and the

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HA tag. The identity of the chimeras was in each case confirmed by DNA sequencing. For transfection experiments the chimeric HA-IκBα genes were recloned into the pRc/CMV eukaryotic expression vector (Invitrogen).

2.2. Cells, transfection and protein analysis

The human cervical carcinoma line HeLa was maintained in Iscovec's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. 70–80% confluent monolayers were transfected with a mixture of 1% w/v plasmid DNA and 0.6% w/v Lipofectamine (Life Technologies) according to standard protocols. The transfected cells were cultured for 48 h before treatment with 100 ng/ml recombinant human TNFα (Boehringer Mannheim). At the indicated time of treatment, the plates were placed on ice and the monolayers were washed with ice-cold phosphate-buffered saline (PBS). Cell lysis, pulse-chase and Western blots experiments were performed as described before [20].

3. Results

3.1. The GAr of EBNA1 contains multiple inhibitory octamer motifs

We have previously shown that insertion of the octamer peptide GGAGAGAG in different positions of IκBα is sufficient to inhibit TNFα-induced proteolysis in vivo [20]. The inhibitory activity was lost when the length of the insert was reduced to four amino acids. Since the inactive inserts, GAGA and GAGG, contained two or one alanine residues, respectively, we hypothesized that the inhibitory motif should contain three or more alanines. The 238 amino acid long full length GAr of the prototype B95.8 EBNA1 (flGA) does not contain adjacent alanine residues. We generated, therefore, a set of all the possible octamers that contain three alanines separated by variable numbers of glycines (Table 1). Octamers containing the same core sequence, Ala–Gly$_n$–Ala–Gly$_n$–Ala,

Table 1
Sequence of predicted GAr octamers containing three alanine residues interspersed by variable numbers of glycines and their representation in EBNA1

Octamer (*x*-*y*)[a]	Sequence[b]	Non-overlapping octamers in EBNA1[c]
1-1	**A**G**A**G**A**GGG	1
	G**A**G**A**G**A**GG	0
	GG**A**G**A**G**A**G	1
	GGG**A**G**A**G**A**	0
1-2	**A**G**A**GG**A**GG	11
	G**A**G**A**GG**A**G	11
	GG**A**G**A**GG**A**	11
1-3	**A**G**A**GGG**A**G	11
	G**A**G**A**GGG**A**	11
1-4	**A**G**A**GGGG**A**	0
2-1	**A**GG**A**G**A**GG	19
	G**A**GG**A**G**A**G	19
	GG**A**GG**A**G**A**	16
2-2	**A**GG**A**GG**A**G	12
	G**A**GG**A**GG**A**	12
2-3	**A**GG**A**GGG**A**	0
3-1	**A**GGG**A**G**A**G	6
	G**A**GGG**A**G**A**	6
3-2	**A**GGG**A**GG**A**	9
4-1	**A**GGGG**A**G**A**	0

[a]The octamers are named according the number of glycines between the first and second (*x*) and second and third (*y*) alanine residue.
[b]Octamers containing the same Ala–Gly$_n$–Ala–Gly$_n$–Ala core motif but different numbers of flanking glycines were considered as synonyms.
[c]The 239 amino acid long GAr of the prototype B95.8-encoded EBNA1 was searched for exact matches to each possible sequence.

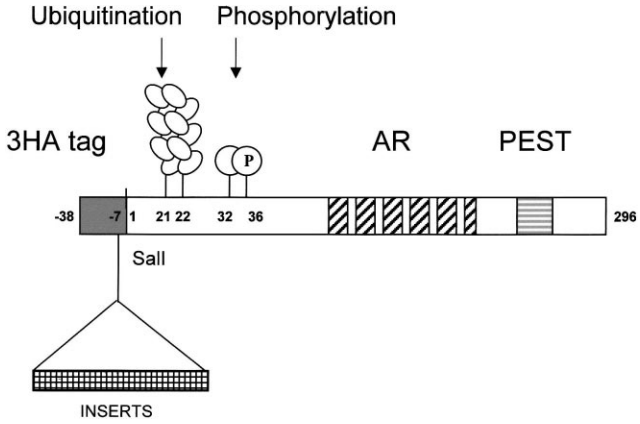

Fig. 1. Schematic representation of the chimeric IκBα constructs. The coding sequence of IκBα is shown as an open box with the five ankyrin repeats (AR) indicated by hatched boxes and the coding region for three influenza hemagglutinin epitopes (HA) indicated by a filled box. The lysines, K21 and K22, and serines, S32 and S36, involved in signal dependent phosphorylation and ubiquitination are indicated. Cassettes encoding repeats of different length and amino acid composition were inserted in frame at the N-terminal end immediately after the HA tag (position −7).

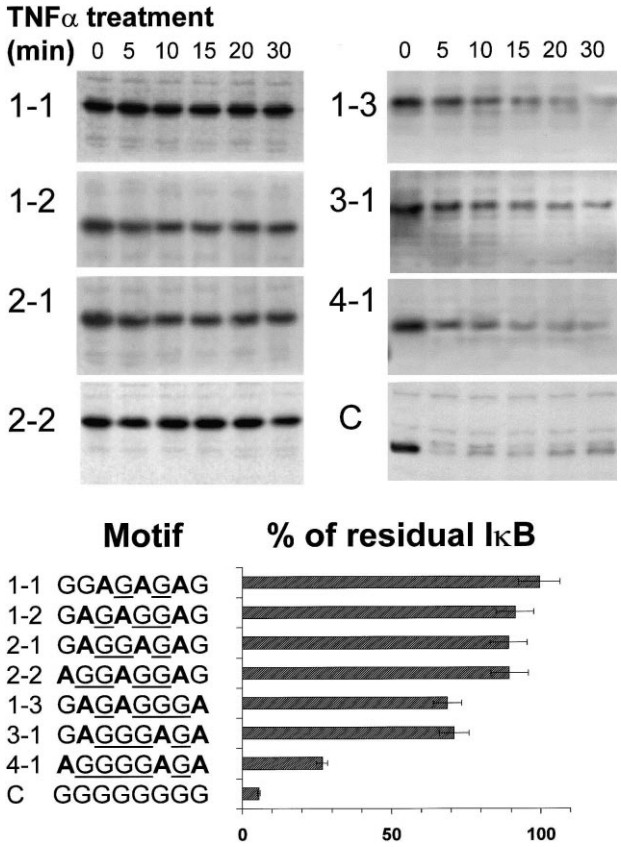

Fig. 2. Inhibition of TNFα-induced proteasomal degradation of IκBα by insertion of GAr octamers with different Gly spacers. HeLa cells transiently transfected with HA-tagged IκB-GAr chimeras were cultured for 48 h before treatment with 100 ng/ml TNFα for the indicated time. A: Western blots of total cell extracts were probed with an anti-HA mAbs. One representative experiment out of three is shown. B: The intensity of the HA-IκB-GAr specific bands was quantified by densitometry. The amount of residual IκB-GAr after 15 min treatment was calculated as the ratio between the intensity of the specific band in treated and untreated cells. Mean ± S.D. of three experiments.

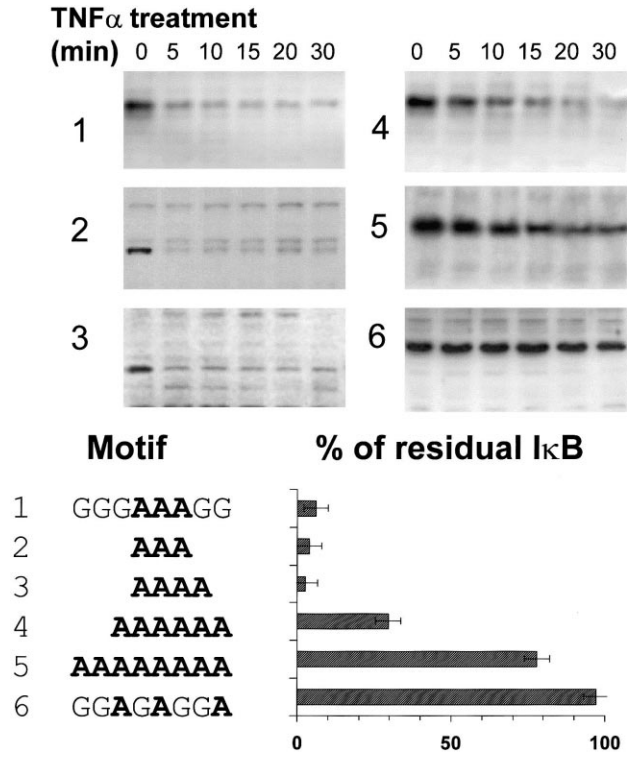

Fig. 3. Gly residues are required for optimal inhibitory activity. HeLa cells were transfected with HA-tagged IκBα chimeras containing the indicated inserts. Poly-alanine inserts of three, four, six and eight amino acids were compared with the prototype inhibitory octamer GAGAGGAG (positive control) and with an eight amino acid long polyglycine (negative control). A: One representative Western blot is shown in the figure. B: Degradation of the chimeras after treatment with TNFα and the amount of residual IκBα after treatment for 15 min were measured as described in the legend to Fig. 1. Mean ± S.D. of three experiments.

but different numbers of N- and C-terminal glycines were considered as synonyms. Only one copy of the GGAGAGA octamer was identified in the flGA sequence using the PatScan algorithm (http://www-unix.mcs.anl.gov/compbio/PatScan/HTML/patscan.htm), whereas the octamers GAGGAGAG, GGAGGAGA, GAGAGGAG, GAGAGGGA, and GAGGGAGA were present 19, 16, 11, 11 and 9 times, respectively (Table 1). Many of the theoretically possible octamers were not found, indicating that there may be restrictions in the expression of these sequences.

A set of hemagglutinin-tagged IκBα chimeras was constructed to test the influence of different EBNA1 derived octamers on the TNFα-induced degradation of IκBα (Fig. 1). Three octamer peptides GAGAGGAG, GAGGAGAG and AGGAGGAG had *cis* stabilizing effects comparable to that of the prototype GGAGAGAG sequence (Fig. 2, panels 1-2, 2-1 and 2-2). A weaker inhibition was observed with the GAGAGGGA and GAGGGAGA peptides (Fig. 2, panels 1-3 and 3-1). Alignment of the inserts suggests that the distance between alanine residues may be important for the inhibitory activity. In accordance, a chimera containing a N-terminal octamer where the first and second alanine residues were separated by four glycines showed weaker inhibitory activity only marginally stronger than that obtained by insertion of a control eight amino acid long glycine polypeptide (Fig. 2, panel 4-1). We next asked whether the spacing itself might be important for the effect. Insertion of three alanine residues, or of an octamer containing three consecutive alanines flanked by glycines, did not prevent TNFα-induced proteolysis (Fig. 3, panels 1 and 2). Polyalanine inserts of four or six amino acids were also inactive whereas partial protection was achieved with an eight alanine long insert (Fig. 3, panels 3–5).

3.2. Hydrophobic residues are required for the inhibitory effect

Additional experiments were performed to evaluate the impact of hydrophobicity on the stabilizing effect of the repeats. Substitution of the three alanines in the peptide GGAGAGAG with the polar serine residues abolished the protective activity (Fig. 4). In accordance with the predicted requirement of at least three alanine residues, the inhibiting activity was lost already after the substitution of a single alanine for serine. Similarly, substitution of the central alanine in the peptide AGGAGGAG with the charged residue aspartic acid abolished the protective activity. We then tested whether alanine could be substituted by other hydrophobic amino acids. As shown in Fig. 4, the inhibitory activity was maintained when all the alanines were changed to the next residue in the scale of hydrophobicity, valine. The GGVGVGVG chimera was as stable as the chimera containing the prototype inhibitor sequence.

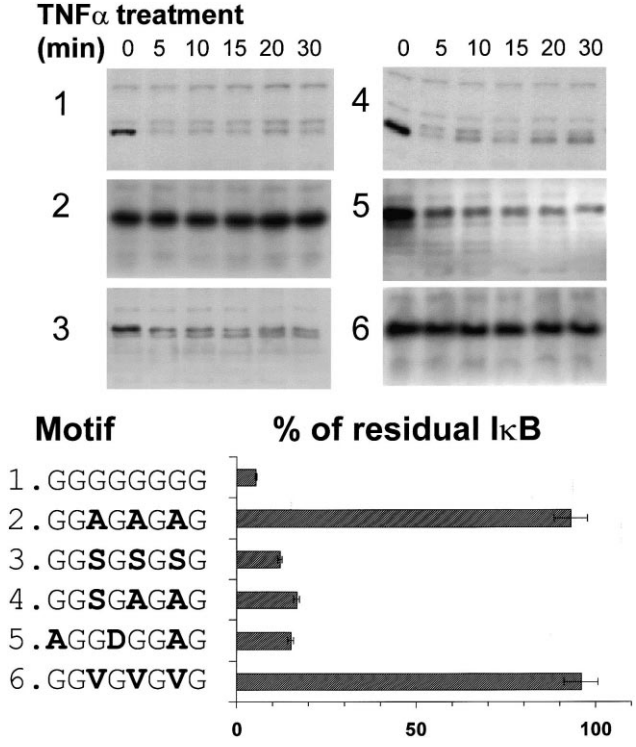

Fig. 4. Hydrophobic amino acids are required for the inhibitory activity. HeLa cells were transfected with IκBα chimeras containing octamers where one Ala residue was substituted by Ser (4) or Asp (5) or all three Ala were substituted with Ser (3) or Val (6). The non-inhibitory polyglycine (1) and the GGAGAGAG octamer (2) served as negative and positive controls, respectively. A: One representative Western blot is shown in the figure. B: Degradation of the chimeras after treatment with TNFα, and the amount of residual IκBα after treatment for 15 min, were measured as described in the legend to Fig. 1. Mean ± S.D. of three experiments.

3.3. Length dependent inhibition of IκBα turnover by variant repeats

In addition to signal dependent proteolysis, the ubiquitin–proteasome pathway is also implicated in the physiological turnover of several cellular proteins, including IκBα (reviewed in [23,24]). This type of regulated proteolysis proceeds with a significantly shower kinetics, probably due to the involvement of a different family of ubiquitin conjugating enzymes and slower rate of ubiquitination [25–29]. In accordance with previous observations [20], the half-life of IκBα, was approximately 30 min while introduction of the GAGAGGAG repeat resulted in a half-life in excess of 12 h (Fig. 5, and data not shown). In accordance with the failure to prevent TNFα-induced proteolysis, the physiological turnover of IκBα was not altered by insertion of the GGSGAGAG sequence. However, significant stabilization was induced by insertion of a longer polypeptide containing three GGSGAGA motifs. We finally tested whether increasing the length of the repeat could compensate the detrimental effect of the serine substitution on TNFα-induced degradation. As expected, an IκBα chimera

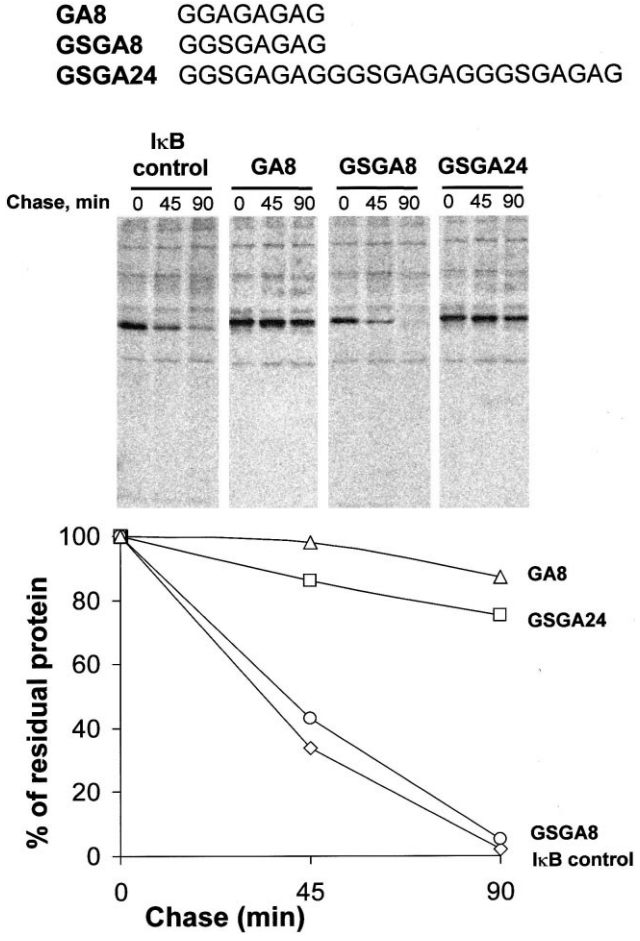

Fig. 5. Impact of length on the inhibitory activity of variant repeats containing Ser residues. Transfected HeLa cells were metabolically labelled and then chased in isotope free medium for the indicated time before immunoprecipitation with the anti-HA antibody and separation by SDS–PAGE. The amino acid sequences of the inserts are shown on the top. A: Phosphorimager images of dried gels from one representative experiment where all the chimeras were tested in parallel. B: The amount of residual IκBα was calculated for each time of chase as the ratio between the intensity of the band at the indicated time of chase and the intensity at time 0.

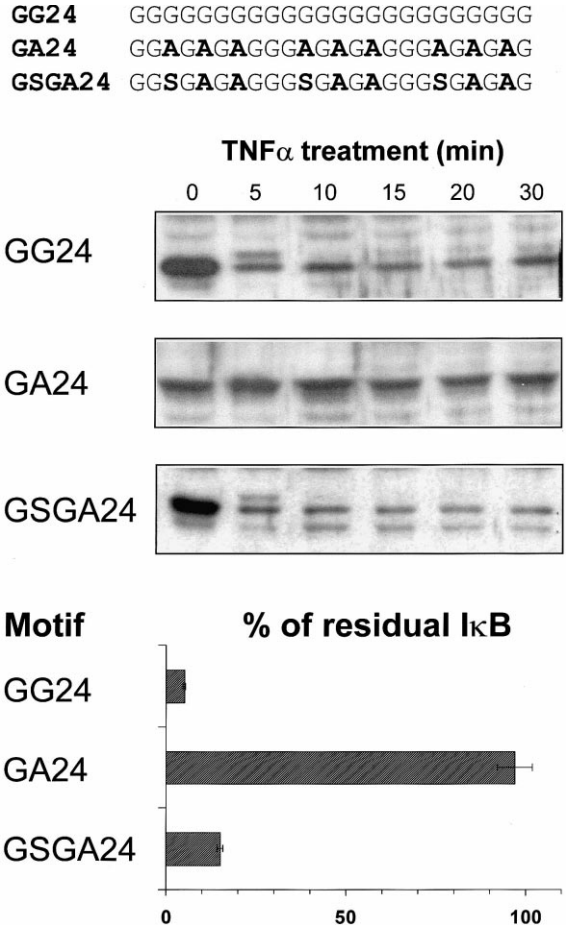

Fig. 6. A long serine containing repeat does not protect IκBα from TNFα-induced proteolysis. HeLa cells transiently transfected with HA-tagged IκBα chimeras containing the indicated inserts were cultured for 48 h before treatment with 100 ng/ml TNFα. A: Western blots of total cell extracts probed the anti-HA mAbs. One representative experiment out of three. B: The intensity of the specific bands was quantified by densitometry. The amount of residual IκBα after 15 min treatment was calculated as the ratio between the intensity of the specific band in treated and untreated cells. Mean ± S.D. of three experiments.

containing a 24 amino acid long polypeptide with three GGAGAGAG motifs was completely resistant to TNFα-induced degradation (Fig. 6). In contrast, insertion of a serine containing repeat of similar length had no significant effect resulting in an half-life only marginally longer than that achieved by insertion of a 24 residue long polyglycine.

4. Discussion

The GAr of the EBV nuclear antigen EBNA1 is the first example of protein domain that acts as a *cis*-inhibitor of ubiquitin–proteasome dependent proteolysis. We have previously shown that an eight amino acid long GAr inhibits the TNFα-induced degradation of IκBα by preventing stable interaction of the ubiquitinated substrate with the proteasome [20]. In the present study, we found a clear relationship between the distance of the three key alanine residues and the inhibitory effect. We have also found that a small hydrophobic amino acid, such as valine, can substitute for alanine without loss of inhibitory effect. The need of three hydrophobic

amino acids was further confirmed by the deleterious effect of substitution of a single alanine with serine. The effect of this substitution was partially compensated by increasing the length of the repeat. Remarkably, however, the longer serine containing repeat could prolong the half-life of IκBα in untreated cells but did not protect IκBα from TNFα-induced proteolysis.

A synthetic eight amino acid long GAr assumes a random coil conformation in solution and lacks a rigid three-dimensional structure upon fusion to IκBα [30]. It is possible, however, that its folding may be stabilized by binding to a putative receptor. The simplest and most attractive model predicts that the binding domain of the GAr should position its hydrophobic residues into three adjacent hydrophobic binding pockets of the receptor. This can be achieved with repeats containing glycine spacers of different length if the peptide is bound in an extended β-strand conformation. When the hydrophobic residues are separated by single glycines the peptide may form a regular β-strand where the side chains of the three hydrophobic residues point in the same direction, resulting in a distance between the marginal methyl groups of approximately 6.3 Å (Fig. 7A). Longer glycine spacers can be accommodated in, so-called, β-bulge structures (Fig. 7B,C). Such β-bulges are found in a variety of proteins [31] and are in some cases functionally important [32]. For β-bulges containing two or three glycines, the adjacent side chains of the hydrophobic residues will be positioned at approximately the same distance as in regular β-strands whereas the distance is significantly increased in β-bulges containing four residues. It is noteworthy that, while octamers containing three alanines spaced by one, two or three glycines are present in multiple copies in the GAr of the prototype B95.8 EBNA1, the 238 amino acid long sequence does not contain four consecutive glycines. Thus all the possible octamers found in the EBNA1 GAr have the capacity to expose their alanine side chains to the same hydrophobic receptor site if the fragments bind in an extended β-strand conformation with β-bulges accommodating spacer regions of one, two or three glycines.

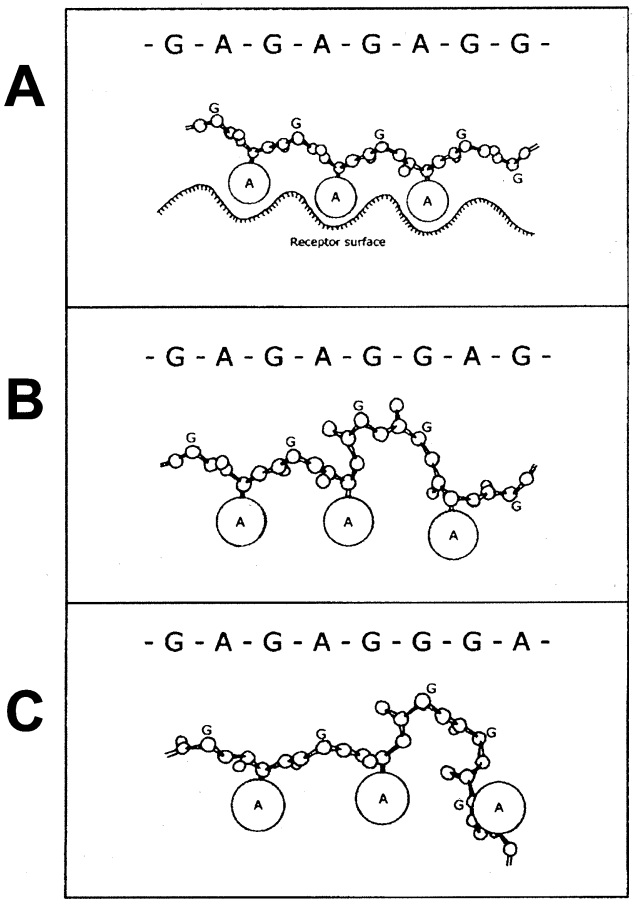

Fig. 7. Possible conformation of the GAr bound to a putative receptor. The flexible nature of the GAr may allow specific interaction with a putative receptor containing multiple hydrophobic pockets. GAr with single glycine spacers may assume a regular β-strand conformation with a distance between the external methyl groups of the hydrophobic residues of approximately 6.3 Å (A). The same distance between the external methyl groups can be achieved if spacers of two (B) or three (C) glycines are accommodated in β-bulge structures. Spacers of four glycines would not allow proper spacing of the methyl groups. Substitution of one hydrophobic residue would significantly weaken the interaction but the effect may be restored by increasing the length of the repeat if more then three hydrophobic pockets are available on the receptor.

In accordance with the prediction that at least three hydrophobic pockets of the putative receptor must be occupied in order to achieve inhibition, the GGSGAGAG peptide failed to prevent the degradation of IκBα in TNFα-treated cells. Interestingly, the effect of serine substitution could be partly compensated by increasing the length of the sequence. A multimer of GGSGAGA sequence prevented the degradation of IκBα in untreated but not in TNFα-treated cells. Conceivably, putative receptor may contain more than three hydrophobic pockets. Occupation of three, non-consecutive pockets may be sufficient to attain low affinity interactions that counteract 'weak' but not 'strong' degradation signals. We have recently observed a similar phenomenon using green fluorescent proteins (GFP) chimeras targeted for proteolysis by N-end rule and ubiquitin fusion degradation (UFD) signals of different strength, as judged by the half-life of the chimeras [33]. Chimeras carrying weak degradation signals were efficiently protected by short GAr sequences, while longer sequences were required to protect chimeras that are degraded with a rapid turnover. The GGSGAGA peptide is the core unit of a 54 amino acid long repeat found in the EBNA1-like protein of the γ-herpesvirus from baboon (baLCV) [34] and a similar serine containing repeat is also found in the γ herpesvirus from rhesus monkey (rhLCL) [35]. Consistent with the possibility that the serine containing repeats may not fully prevent proteolysis, substitution of the EBNA1 GAr with the baGAr or rhGAr repeats did not prevent the presentation of epitopes from EBNA1 to MHC class I restricted CTLs [16]. This may indicate that protection from CTL recognition is not a conserved function of the repeats. Alternatively, the presence of serine residues in the GAr-like sequences of primates may reflect the adaptation to different combinatorial surfaces found in the putative receptors of the endogenous species. It is noteworthy that a long repeat is also present in LANA of the second known human γ-herpesvirus, HHV8 [17,18]; the function of this repeat is presently unknown.

The major known function of EBNA1 is the maintenance of EBV episomes in proliferating virus-infected cells [36–38]. EBV-infected normal B-cell blasts express, in addition to EBNA1, numerous viral antigens that are efficiently recognized by EBV-specific immune responses [39–43]. It is, therefore, unlikely that failure to present epitopes from EBNA1 would significantly contribute to their escape from T-cell sur-

veillance. Conceivably, inhibition of EBNA1 proteolysis by the GAr may be important in other types of virus–host relationships that are not recapitulated by infection of primary B-lymphocytes in vitro. It is also possible that the stabilizing effect of the GAr may serve a non-immunologic purpose. Although only a minority of freshly separated EBV carrying 'resting' B-lymphocytes appear to express EBNA1 mRNA [44], a stable EBNA1 protein could persist in these non-proliferating cells in the absence of transcription.

Clearly, much remains to be done to clarify the function of the GAr in the context of EBV infection. In addition, the capacity of the GAr to inhibit proteasomal degradation in *cis* provides an interesting new tool for the selective stabilization of proteins that are degraded by the ubiquitin–proteasome pathway in vivo. Protection of transduced proteins from proteasomal degradation, in order to prolong their half-life or prevent immune recognition, remains an important task for gene therapy.

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